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TITLE: ANTIBODY-FUNCTIONALIZED CARBON NANOTUBE TRANSISTORS AS BIOSENSORS FOR THE DETECTION OF PROSTATE CANCER

PRINCIPAL INVESTIGATOR: A.T. Charlie Johnson, Ph.D.

CONTRACTING ORGANIZATION: University of Pennsylvania,
Philadelphia PA 19104

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A.T. Charlie Johnson, Ph.D.
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14. ABSTRACT
During year 1 of this award we successfully met nearly all benchmarks proposed in our joint statement of work. This was accomplished through active interaction between the two groups. Face-to-face meetings, videoconferences, and numerous email communications and trips between sites to deliver reagents allowed for useful discussions and insured rapid research progress. The Johnson group successfully fabricated and tested swCN transistors with covalently bound scFv antibodies specific for to one of our proof-of-concept biomarkers that had been developed by the Robinson group. We are now in position to begin testing sensitivity of our swCN sensors and developing optimized strategies for functionalizing the sensors with scFv antibodies.

The ultimate goal of this research is the development of a prototype biosensor that will be used to retrospectively test patient samples during year 3 of this award. The research accomplishments achieved in year 1 have positioned us well for meeting this goal. Assuming our hypothesis of increased sensitivity over current technology is correct, by the end of this funding period we aim to have generated sufficient data to support the development of this sensor platform.

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Year 1 Report for Contract W81XWH-09-1-0206

ANTIBODY-FUNCTIONALIZED CARBON NANOTUBE TRANSISTORS AS BIOSENSORS FOR THE DETECTION OF PROSTATE CANCER

PI – A.T. Charlie Johnson, Jr.

PCRP SYNERGISTIC IDEA DEVELOPMENT AWARD, JOINT PROPOSAL WITH PROPOSAL LOG NUMBER PC080542, FOX CHASE CANCER CENTER, DR. MATTHEW ROBINSON. THE LINKED GRANT AWARD IS W81XWH-09-1-0205.

Introduction

Early detection and differential diagnosis are critical components for the successful treatment of prostate cancer (CaP). The research being carried out under this Synergistic IDEA Award is focused on the development of a novel biosensor platform for the detection of CaP biomakers in biologic fluids, such as

serum. The proposed biosensors are comprised of antibody-functionalized single-wall carbon nanotube (swCN) transducers. We hypothesize that the specificity inherent in antibody-antigen interactions, when combined with the swCN platform, will create a novel biosensor platform with increased sensitivity over currently available technologies. The goals for this three year funding period encompass the generation of swCN and single-chain antibody (scFv) reagents necessary to carry-out initial proof-of-principle experiments (year 1), optimizing the biosensors for use in biologic fluids (year 2), and performing a retrospective analysis of patient samples to determine the sensitivity and specificity of the biosensors (year 3).

Body

Research as been carried out all three aims of this grant during months 1 – 12 of this grant period, as defined by the original statement of work. Progress achieved toward the successful completion of each of these aims is listed in detail below.

Aim 1. Construction and initial validation of a single-wall carbon-nanotube biosensor for detecting prostate cancer

Year one goals of Aim 1 include the development of high quality fabrication techniques for carbon nanotubes transistors; methods for functionalizing them with single-chain Fv antibodies (scFv) to prostate cancer biomarkers that are developed as part of this project (see Aim 2 for details); and testing of nanotubes sensors against common interferents such as salt and serum albumin. We have made significant progress along each of these research directions. The experiments discussed below relied on the use of single chain variable fragments antibodies (scFv) specific for osteopontin (OPN), one of the three biomarkers of interest to this proposal. The scFvs were developed by our project partners in the Robinson group at Fox Chase Cancer Center.

Carbon nanotubes are synthesized by catalytic chemical vapor deposition, and then integrated into transistor devices using photolithography, a process that is the basis of modern large-scale semiconductor device manufacturing. In this reporting period we have developed new methods suitable for covalent functionalization of nanotube transistors with antibodies at very high yield, roughly 80% in our latest experiments. The new approach relies on the use of carboxylated diazonium salts, which spontaneously form covalent bonds to carbon nanotubes at elevated temperature [1]. A mild diazonium treatment is used, since excessive covalent modification of nanotubes destroys the semiconducting properties necessary for efficient signal transduction [1,2]. The carboxylic acid functionality of the diazonium salt is activated with a standard EDC/sNHS treatment. At this point we have a choice as outlined in the next two paragraphs.

The first option is to attach a nitrilotriacetic (NTA) linker. This treatment results in roughly 1 attachment site for each 150 nm length of exposed nanotube, as seen in Figure 1(b). Device fabrication is completed with the addition of Ni ions, which are chelated by the NTA complex, and then incubation in a solution of His-tagged antibodies that associate with the Ni-NTA attachment sites on the devices. This approach gives control over the antibody orientation since a polyhistidine tag can be engineered into the antibody, as is commonly done to aid in protein purification [3]. We expect this approach to be ideal for detection of OPN in biological samples. However, it is not appropriate for our recent experiments (described below) done on samples spiked with recombinant OPN, since these OPN molecules also have a His-tag, and so would potentially bind to the NT device through the Ni-NTA linker. Results from

attachment experiments with His-tag labeled gold particles and His-tagged proteins are shown in Fig. 1.1.

The second attachment procedure is to simply incubate in protein after the activation with EDC/sNHS. This results in an amide bond formed between the protein and the nanotube with only partial control over the protein orientation since bonding can occur at amino acids amino acids Arg, Asn, and Gln and at the N-terminus of the protein backbone [4]. We have implemented this using scFvs to OPN and verified the attachment by AFM and electronic measurement (Fig. 1.2). We have also begun experiments to quantify the sensor response to solutions of OPN. Fig. 1.2c shows data how the current-gate voltage (I-Vg) characteristic of the device is altered after exposure to OPN in buffer at a concentration of ~500 ng/mL. This response was replicated in 3 different devices. In the coming period we will determine how the device response varies with OPN concentration, and also conduct a variety of negative controls to validate device specificity.

We have also tested the response of NT transistors to interfering compounds, specifically salt-containing buffer and serum albumin. We find that there is a significant response in both cases, which we ascribe to charge screening in the case of buffer and non-specific adsorption for albumin. For buffer, the response can be made negligible by dilution to a salt level below about 10mM. This suggests that desalting procedures will be required for reliable measurement of biomarkers in serum. The response to albumin is a more significant issue, and in the coming period we will investigate alternative approaches to suppressing unwanted protein absorption in this system.

Aim 2. Optimizing antibodies to enhance biosensor capabilities

The year one goals set forth in the statement of work for Aim 2 include the isolation of single-chain Fv antibodies (scFv) specific for prostate specific antigen (PSA) urokinase plasminogen activator receptor (uPAR), and osteopontin (OPN), the three biomarkers of interest. Once isolated and characterized, initial engineering steps to enable site-directed conjugation were proposed. As outlined below each of these steps has been achieved.

Specificity of the biosensors will be dictated by the antibody that is conjugated to the swCN. To facilitate detection of the CaP biomarkers PSA, uPAR and OPN we have successfully isolated scFv antibodies specific for each of these proteins. Intact monoclonal antibodies (mAbs) with appropriate epitopes and binding characteristics making them suitable for detection of the biomarkers in serum are described in the literature and co-crystal structures of a subset are publicly available (Figure 2.1). We have taken advantage of co-crystal structure data of ATN-615 (anti-uPAR), 23C3 (anti-OPN), and 8G8F5/2ZCL to create scFv versions of each mAb. Single-chain Fv antibodies are ~25kDa proteins comprised of the antigen binding domains of a mAb [variable heavy (Vh) and variable light (Vl) domains] joined through a flexible peptide linker. Using the publicly available protein sequences we synthesized genes encoding scFv versions of each mAb. Genes, codon-optimized for expression in *E. coli*, were synthesized on NcoI/XhoI fragments in the Vh-linker-Vl orientation using the classic (Gly_4Ser) scFv linker and sub-cloned into the pSYN2 bacterial expression vector inframe with the vector encoded 6xHIS tag to facilitate IMAC purification. Each of the scFv were expressed, purified, and binding activity characterized via surface plasmon resonance (SPR) using a BIAcore1000. The ATN-615 and 23C3 scFv were purified by sequential IMAC and size exclusion chromatography and then analyzed by SPR (Figures 2.2 and 2.3). Gel filtration fractions containing purified scFv were pooled and a kinetic analysis of binding was performed using CM5 chips coated with commercially available

antigens. Theoretical Rmax for the two surfaces were 384 (uPAR) and 115 (OPN) RU. ATN-615 binds to uPAR with an affinity of 7.9 x 10^{-10} M and 23C3 binds to OPN with an affinity of 2.9 x 10^{-10} M. The Observed Rmax for both surfaces was consistent with surfaces that were at least 90% active. In contrast to ATN-615 (Figure 2.4 right panel) and 23C3 (data not shown), which chromatographed over a Hi Prep 16/60 Sephacryl S-100 size exclusion column as a single peak of a size consistent with the 25 kDa protein 8G8F5/2ZCL scFv purified as three distinct peaks ranging in size from 25 kDa and larger. (Figure 2.4 left panel). When analyzed by SDS-PAGE peaks resolved as a single 25 kDa protein (Figure 2.5). This data is consistent with the 8G8F5/2ZCL scFv forming higher-order aggregates, a known issue with some scFv. Fractions containing monomeric 8G8F5/2ZCL bound to recombinant PSA (Figure 2.5) as judged by SPR (theoretical Rmax = 164, observed Rmax = 64) (Figure 2.5). Although potentially usable for initial proof-of-concept studies we plan to engineer the framework of the 8G8F5/2ZCL scFv to overcome the aggregation issue. This will be accomplished by engineering a disulphide-stabilized form of the scFv using well-established protocols[5]. We anticipate completing the optimization in the first quarter of the second year of funding.

Instrumental to the success of the antibody-functionalized swCN is the ability to conjugate scFv antibodies in a manner that results in attachment of reproducible levels of active scFv on the sensor surface. An aim 2 goal (months 9 – 18) is to engineer scFv for site-directed conjugation (Figure 2.6). We proposed to conjugate scFv antibodies to the swCN in a site-directed manner through incorporation of a free cysteine residue into the scFv to facilitate either thiol- or maleimide-based coupling strategies. Appropriate oligonucleotide primers have been ordered to incorporate a cysteine-encoding codon into each of the scFv genes by site-directed mutagenesis. We anticipate the mutagenesis and impact of this alteration on scFv stability and function will be completed by the end of the first quarter of year 2. Simultaneously, as part of other work we have developed the ability of to conjugate proteins to swCN through the 6xHIS purification tag present at the C-terminus of our scFv. This provides us with a universal strategy to couple scFv in an oriented fashion without the need for additional modification steps that could deleteriously affect the stability or antigen binding capabilities. Thus, although we will continue to pursue the cysteine-based methods we have developed technology that allows site-directed conjugation.

In addition to providing a uniform surface, site-directed conjugation will allow us to test our hypothesis that decreasing the distance between the antigen binding domain and the swCN surface will increase the sensitivity of the sensor. We proposed to evaluate linkers of different lengths to increase this distance. For other purposes we have begun creating a vector that will allow for expression of C-terminally 6xHIS-tagged Fab fragments in *E. coli*. As depicted in Figure 2.6 the 50 kDa Fab (comprised on variable and first constant domains) doubles the distance between the swCN and antigen binding as compared to the scFv. Once engineered, comparing His-tagged Fab and scFv versions of each antibody via our newly developed His-tag conjugation strategy will provide an alternative to the proposed chemical conjugation strategies.

<u>Aim 3. Validation of swCN biosensors in patient samples</u>

To enable our access to serum samples from CaP patients and normal volunteers that are housed in the Fox Chase Cancer Center biosample respository an exempt protocol was prepared and submitted for approval by the FCCC IRB. This protocol has been approved and we now have access to all of the necessary serum samples to carry out our proposed work. A copy of the approval letter is appended to the report submitted by partner PI Matthew Robinson.

Key Research Accomplishments

- Isolated functional scFv antibodies against each of the three target antigens
    - Targets are osteopontin (OPN), Urokinase Plasminogen Activator Receptor (uPAR) and prostate specific antigen (PSA)
- Measured binding affinity for two of the three scFv by surface plasmon resonance
    - 23C3 and ATN-615 bind to OPN and uPAR, respectively, with sub-nanomolar affinity
- Site-directed conjugation of scFv to swCN surface
    - Developed novel strategy to conjugate scFv to swCN through 6xHIS purification tag present on the C-terminus of scFv
    - Designed and ordered reagents necessary to incorporate cysteine residue into the linker region of scFv, isolated as part of Aim 2.1, to facilitate conjugation via standard thiol chemistry.
    - Two approaches to site-directed conjugation of scFv to the swCN will retire risk associated with this critical step in sensor development.

Reportable Outcomes

- Site-directed conjugation of scFv to swCN surface was included in invited oral presentations at multiple international conferences: Center for Integrated NanoTechnologies (CINT) User Meeting, Albuquerque, New Mexico, August 2010; Nanotube 2010 (NT10) meeting, Montreal, Canada (June 2010), Gordon Research Conference on Physics Research and Education, South Hadley, Massachusetts, June 2010; "Nano Helps Bio" Conference, Santa Fe, New Mexico, April 2010. A manuscript is in preparation.
- Final IRB approval was obtained for clinical protocol to obtain CaP samples from the FCCC biosample repository
- This research led directly to a new collaboration between the initiating PI (Dr. Matthew Robinson) and Dr. Gianluca Piazza (University of Pennsylvania, Department of Electrical and Systems Engineering) to develop a second type of sensor platform. Research is focused on detection of immune modulatory molecules related to CaP.
    - Collaboration resulted in the successful application for external pilot funding to support the development of this second sensor platform.
    - Additional funding applications have also been submitted
- Research results were presented in poster format at 15th Annual Postdoctoral and Graduate Student Research Symposium, FCCC.

Conclusions

During months 1 – 12 of this synergistic idea award we have successfully met most, if not all, benchmarks set forth in our statement of work for both research groups. This has been accomplished through an active interaction between the Robinson and Johnson groups. Two face-to-face meetings and two videoconferences, along with numerous email communications and trips between sites to deliver reagents, allowed for critical discussions related to research goals and insured that research progress was maintained. We have both successfully isolated the scFv antibodies specific for each of our proof-of-concept biomarkers and had initial successes with testing of antibody-functionalized swCN in both

aqueous buffers and simulated serum. Thus, we are now in position to begin testing sensitivity of our swCN sensors and developing optimized strategies for functionalizing the sensors with scFv antibodies.

The ultimate goal of this research is the development of a prototype biosensor that will be used to retrospectively test patient samples during year 3 of this award. The research accomplishments achieved in year 1 have positioned us well for meeting this goal. Assuming our hypothesis of increased sensitivity over current technology is correct, by the end of this funding period we aim to have generated sufficient data to support the development of this sensor platform.

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(1) Bahr, J. L.; Yang, J. P.; Kosynkin, D. V.; Bronikowski, M. J.; Smalley, R. E.; Tour, J. M. *Journal of the American Chemical Society* **2001**, *123*, 6536-6542.

(2) Graff, R. A.; Swanson, T. M.; Strano, M. S. *Chem Mater* **2008**, *20*, 1824-1829.

(3) Raming, K.; Krieger, J.; Strotmann, J.; Boekhoff, I.; Kubick, S.; Baumstark, C.; Breer, H. *Nature* **1993**, *361*, 353-356.

(4) Johnson, R. R.; Rego, B. L.; Johnson, A. T. C.; Klein, M. L. *Journal of Physical Chemistry B* **2009**, to be published. Cover article.

(5) Worn, A.; Pluckthun, A. *J Mol Biol* **2001**, *305*, 989-1010.

Supporting Data

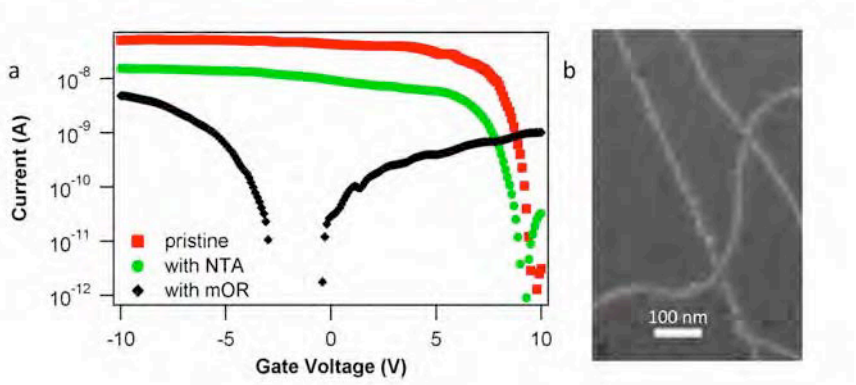

Figure 1.1 I(Vg)s of a carbon nanotube device in its pristine state (red squares), after diazonium-NTA-Ni functionalization (green circles) and after attachment of His-tagged mouse olfactory receptors (black diamonds). Bias voltage is 100 mV in all cases. (b) SEM image demonstrating attachment of His-tag labeled 30nm gold nanoparticles to carbon nanotubes using diazonium-NTA-His chemistry.

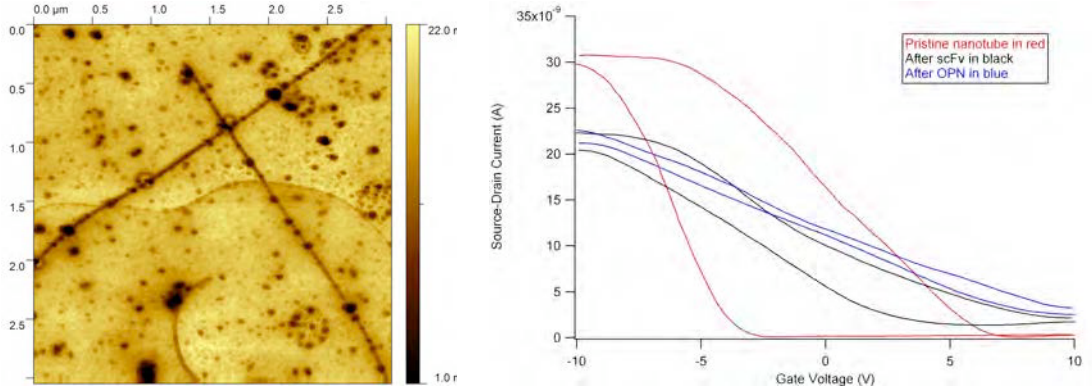

Figure 1.2 a) AFM image showing attachment of proteins to carbon nanotubes using the diazonium carboxylated diazonium salt/EDC/sNHS chemistry. b) I-Vg characteristics of an as-grown carbon nanotube transistor (red), after fucntionalization with scFv to OPN (black) and upon exposure to a solution of OPN at a concentration of 500 ng/mL (blue) in 0.5M phosphate buffer solution diluted 1:100 with deionized water. A clear response is observed, suggesting that the detection scheme is effective.

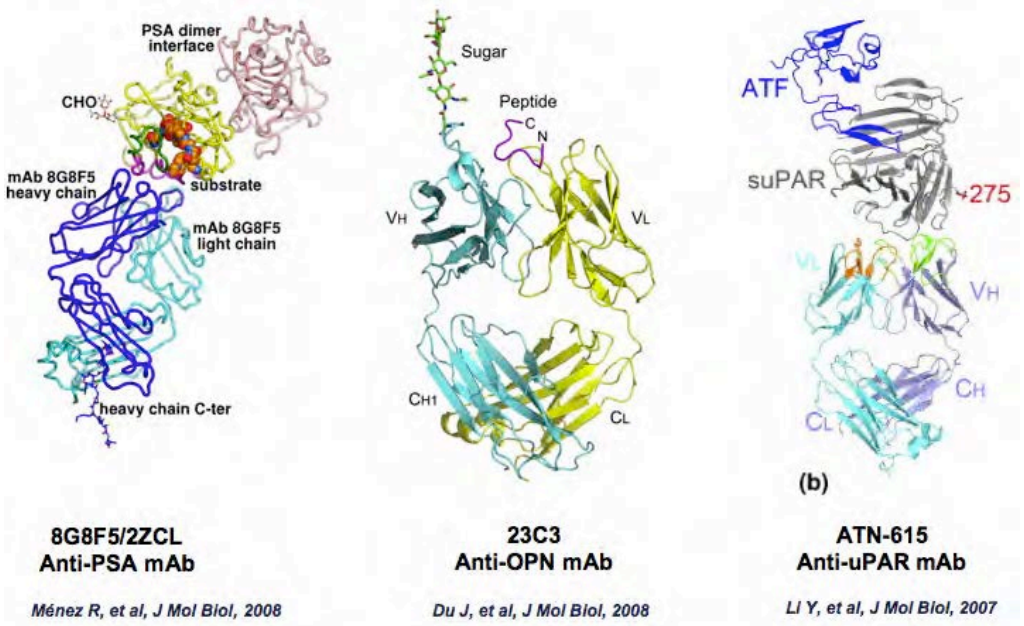

Figure 2.1. Co-crystal structures of relevant antibody Fab fragments bound to biomarkers of interest. Monoclonal antibodies specific for biomarkers of interest were identified in the literature. Co-crystal structures depicted above demonstrate that the mAb bind appropriate epitopes to be used for detection of the antigens in serum.

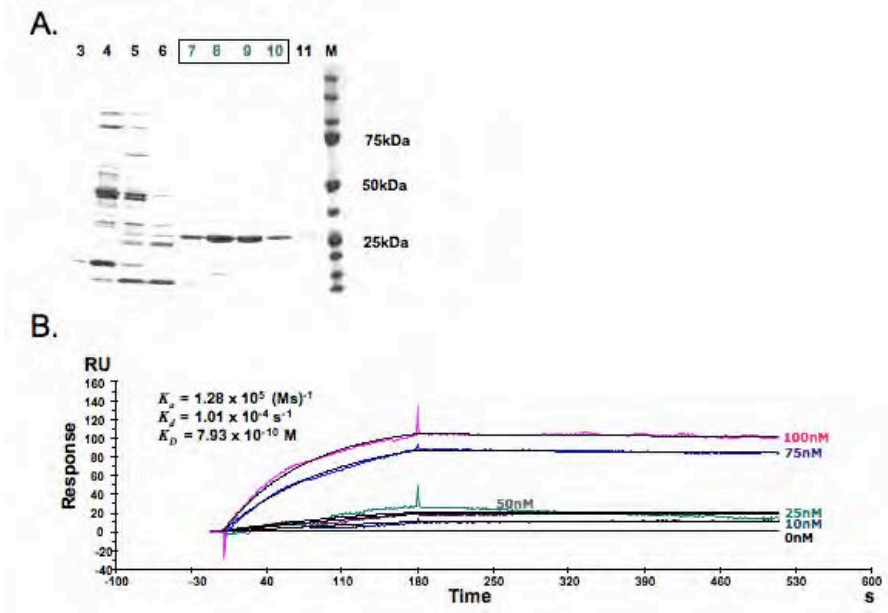

Figure 2.2. Characterization of the anti-uPAR ATN-615 scFv
A) SDS-PAGE analysis of IMAC purification over Ni-NTA column. Fractions 7 – 10 were pooled, subjected to gel filtration chromatography. B) Gel filtration fractions containing purified scFv were analyzed by SPR and kinetic constants determined with BIAevaluation using a 1:1 binding model and global fit analysis.

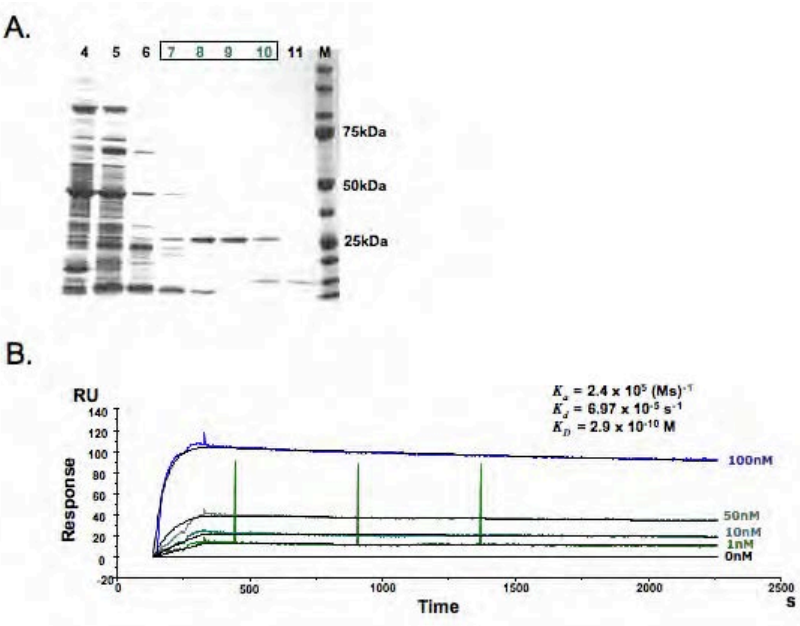

Figure 2.3. Characterization of the anti-OPN 23C3 scFv
A) SDS-PAGE analysis of IMAC purification over Ni-NTA column. Fractions 7 – 10 were pooled, subjected to gel filtration chromatography. B) Gel filtration fractions containing purified scFv were analyzed by SPR and kinetic constants determined with BIAevaluation using a 1:1 binding model and global fit analysis.

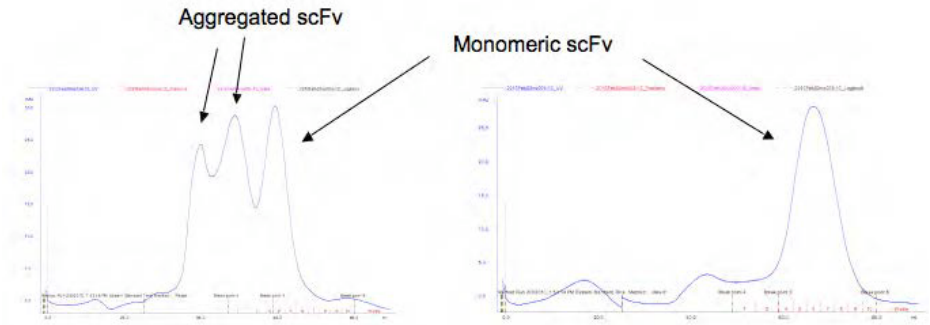

Figure 2.4. Gel filtration analysis of 8G8F5/2ZCL and ATN-615 scFv
The 8G89F5/2ZCL (left panel) resolved as three distinct peaks as compared to the single peak seen with theATN-615 scFv (right panel).

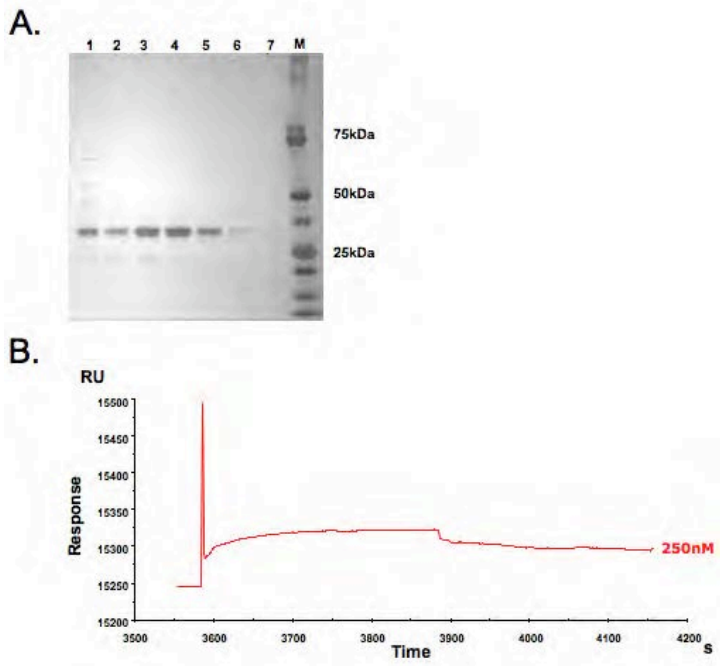

Figure 2.5. 8G8F5/2ZCL scFv
A) SDS-polyacrylamide gel electrophoresis analysis of S-100 gel filtration fractions across all three peaks, suggesting that high molecular weight peaks are aggregated forms of the scFv. B) Monomeric 8G8F5/2ZCL binds to recombinant PSA (R&D) as analyzed by SPR

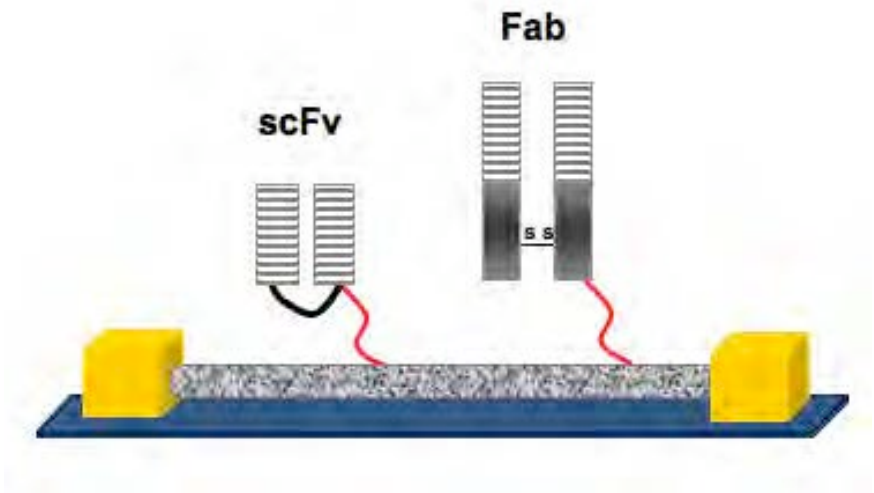

Figure 2.6. Schematic of scFv and Fab conjugated to swCN via site-directed conjugation.
Site-directed conjugation of a Fab fragment would increase the distance of antigen binding site (oriented away from swCN in cartoon) from the swCN by 2-fold facilitating the testing of our hypothesis that as distance increases sensitivity of the detector decreases. Antibodies not drawn to scale with swCN.